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INFLUENCE OF SOIL POPULATIONS of ASPERGILLUS FLAVUS ON AFLATOXIN CONTAMINATION OF CORN

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3. Executive Summary:

Project Title I. Determination of the heterokaryon compatibility group pattern in a field containing an endemic population of *Aspergillus flavus*.

Aspergillus flavus group species were isolated from soil samples collected throughout the Philippines and from at least two fields on each major island. *Aspergillus flavus* populations (propagules/gram) were assessed by following standard microbiological techniques and using the spread plating method. In general, soils from VISCA, Baybay, Leyte exhibited the heaviest load of *A. flavus* propagules (208,927) propagules/g) followed by soils collected from IPB-CMU Research Station Musuan, Bukidnon (8,314 propagules/g).

The *A. Flavus* group species were categorized as *A. tamarii, A. parasiticus* and *A. flavus*. The *A. flavus* strains were further classified into small (S) strain and large (L) strain to provide an improved basis for selection and characterization of *A. flavus* strains to be released in agricultural fields. It is apparent that *A. flavus* strains were present throughout the country and that L strains are most common and occur frequently compared to S strains. Screening of *Aspergillus flavus* group for production of aflatoxin was conducted. The results suggest that aflatoxin production by different *A. flavus* isolates is highly variable.

Complementation between nit$^-$ mutants was tested by seeding conidial suspensions in a well 2 cm apart on agar and incubating them for 14 days at 30°C. Each test paired an unknown isolate with two complimentary testers: a NiaD mutant (mutant in the nitrate reductase structural locus) and either a nirA$^-$ (mutant in a regulatory locus governing nitrate and nitrite reduction) or a cnx (mutant in one of several genes for molybdenum-containing

cofactor necessary for nitrate reductase activity). Complementation was indicated by a zone of dense, wild-type growth, often accompanied by sclerotium formation.

Five hundred mutants, isolated from the soil collected all over the country, were tested for growth on different nitrogen sources and classified according to Cove (1976). The niaD mutants were the most frequently occurring followed by NirA$^-$, and least was cnx. Complementation tests revealed that there were 26 VCG's with a total of 150 isolates. It appears that the distribution of vegetative compatibility groups in various fields was significantly different. These results suggest that, although many compatibility groups are widely distributed, a simple field may have a unique population profile.

Project Title II. Establishing a direct relationship between soil populations of *Aspergillus flavus* and aflatoxin contamination of corn.

Conclusive proof that the source of pre-harvest inoculum for corn is the soil requires the fulfillment of Koch's populates. As such, field simulation studies of a marked strain of *A. flavus*, Min1A2, were carried out in Ilagan, Isabela soils, where corn is a major crop grown throughout the year. Two types of inoculation by spraying and basal application were done using different levels of inocula. This was done to keep track of the movement of *A. flavus* from the soil into the infected kernels.

Results revealed that high levels of colonized corn inoculum at 400 g colonized corn applied basally were correlated with a high percent of kernel infection, 26.7%, and toxin production, 176 ppb aflatoxin, B_1 during the dry season. It appears that the higher the

inoculum level and that by applying it basally, then Koch's postulate hypothesis could be illustrated in this experiment.

However, DMRT statistical analysis of the infection on ears among the treatments showed no apparent significant differences. This suggests that there is no correlation between the type of inoculation and levels of inoculum with surface infection on maize seeds. Based on visual observation, the damaged ears can be attributed to *Aspergillus, Fusarium*, insects, particularly borer and earworm, *Penicillium* and nutrient deficiencies.

A. flavus propagule counts of the soil before and after treatment were assessed to determine the diversity of *A. flavus* and to establish the presence of Min1A2 isolates. Soil samples were pressed by soil dilution-spread plating technique using selective modified Rose Bengal Agar. Propagule counts of *A. flavus* from the soil during the dry season were assessed and analyzed. It was observed that propagule counts increased ten-fold after treatment or harvesting. However, high and consistent percent of kernel infection toxin production through these simulations were observed only on treatments with the highest levels of inocula at 200 and 400 g colonized corn inoculated basally. This could be due to the preying of insects, mites, etc. on predisposed surface conidia, mycelia and other vegetative structures made available to them on soil surfaces. It appears that soil mycostasis might have occurred, because it was observed that, when the incidence of *A. flavus* was low, *Fusarium* spp. propagules were high, which explains pre-emptive colonization of *A. flavus* at high levels of inocula.

These findings play a pivotal role in the prevention/control of *A. flavus* onset of infection prior to silking. Therefore, the time of planting, spacing, timing of insecticide treatment and other cultural practices are of concern to effectively manage storage mold problems in general and *A. flavus* in particular.

Project Title III. Determining the importance of insects and wounding to kernel infection of *A. flavus* and what types of wounds predispose corn cobs to infection.

The role of insects and other ear damaging pests in pre-harvest aflatoxin contamination is believed to be one of the predisposing factors to fungal invasion. Wounds or ear damage created by these pests provide an avenue of entrance for the fungus. Further, these insects could also serve as effective dispersal agents. This study was therefore conducted in order to establish a relationship between insects, wounding, *Aspergillus* infection and subsequent aflatoxin contamination of corn.

An experiment with eight treatments and five replications was conducted at Ilagen, Isabela during the 1993 dry season. Plants were wounded and some plots were treated with insecticide to reduce insect activity. Inoculation was done at 20 DAE and wounding was simulated at 50% silking stage. Extent of infection was assessed after harvest by plating kernels on malt salt agar plate, and aflatoxin was extracted using AOAC standard procedures and quantified by thin layer chromatography.

The number of damaged kernel/ear, the extent of *Aspergillus* infection and aflatoxin contamination in both kernels adjacent and away from the wound were relatively higher in the wounded compared to the unwounded treatment. Wounded, inoculated and no insecticide had the highest infection of 46.4% and highest aflatoxin content of 88 ppb for kernels

adjacent to the wound, whereas the unwounded counterpart had only 9.8% infection and 21.6 ppb aflatoxin. When the insecticide was applied, wounded and inoculated, only 30.1% infection was observed, and aflatoxin content quantified was 57.6 ppb. This shows that insects can create the necessary wound or ear injury for the entrance of fungal spores and, at the same time, spread the spores within the ear to further enhance the infection process. Application of insecticide reduces insect activity, thereby causing a subsequent reduction in kernel infection and aflatoxin contamination.

In summary, wounding and insecticide application play an important role in kernel infection and aflatoxin contamination of pre-harvest corn, so that wounded, inoculated, and no insecticide treatment had the highest infection and aflatoxin content.

Rats and lepidopterous pests are among the most serious and destructive pests of corn causing loss in yield. Damage created by these pests may also predispose corn cobs to infection by *Aspergillus flavus* and other fungal pathogens. To determine the influence of wound types created by these pests on kernel infection, a simulation study was conducted. Corn colonized by 10^6 spores *A. flavus*/ml was basally inoculated to 20-day-old seedlings and wounding was done at the 50% silk stage of corn. Another set of treatments involved inoculation after wounding by spraying the corn ears with 10^6 spore suspension *A. flavus*/ml. Kernel infection was determined at harvest by plating on malt salt agar plate, and aflatoxin content was determined by thin layer chromatography.

Results from the 1993 dry season crop showed that infection of kernels around the wound were consistently higher compared to those kernels away from the wound, and, subsequently, aflatoxin content determined also revealed the same trend. Among the

simulated corn borer, simulated rat damage and unwounded treatments, highest infection and aflatoxin content was observed in the simulated rat damage sprayed with *A. flavus* spore suspension a day after wounding, followed by the same kind of damage, but inoculated at seedling stage. The simulated corn borer damage did not vary significantly with the control at both times of inoculation with regards to kernel infection, but aflatoxin content varied significantly with the control when inoculation was done at silking stage. The unwounded, inoculated control was also infected by *A. flavus* which may be attributed to the natural wounds caused by some earworms and corn borers that were not completely controlled by insecticide application. In summary, kernel infection and aflatoxin content were directly proportional to the degree of damage created on the developing corn ears.

4. Research Objectives:

Of the large number of mycotoxins described, aflatoxins have received the greatest amount of attention. Attention has focused on aflatoxin, because it is an animal carcinogen and because it is a common contaminant of many agricultural crops, especially corn. Aflatoxins encompass a large group of related compounds produced by *A. flavus* Link ex Fr. and *A. parasiticus* Spear. These organisms are associated with the *Aspergillus flavus-oryzae* group. They are characterized by the production of yellow-green conidia and the absence of ascospores (Raper and Fennell, 1965). Taxonomically, *A. parasiticus* and *A. flavus* are nearly identical. Subtle morphological differences are utilized to distinguish between the two organisms. Both *A. flavus* and *A. parasiticus* have the ability to produce aflatoxin. Generally, *A. parasiticus* produces aflatoxin B_1, B_2, G_1, and G_2, while *Aspergillus flavus* produces only aflatoxin B_1 and B_2. In addition, many other aflatoxins have been isolated from fungal cultures. Biologically reduced and hydroxylated aflatoxins such as aflatoxins Q_1, H_1, and P_1 have been isolated (Maggon et al., 1977).

Spores from an infected crop are returned to the soil upon harvest. Here, the spores overwinter to provide the inoculum for the next year's crop. No documentation, however, exists demonstrating that the soil is the source of inoculum. In addition, the relative importance of conidia and sclerotia is not known. Sclerotia are much more resistant to adverse environmental conditions compared to conidia. On the other hand, conidia are more numerous in soil than sclerotia.

Many crops have been observed to be infected by *Aspergillus flavus* and *parasiticus*. Each year, more and more crops are reported to have been infected by these organisms. Important crops which are infected include corn, small grains, peanuts, cottonseed, cassava, copra, and a variety of nut crops and spices (Diener and Davis, 1969).

The toxicity of aflatoxin in feed and food has been difficult to accurately determine; however, it is generally believed that aflatoxin is both highly toxic and extremely carcinogenic. The difficulty in measuring the exact effect of aflatoxin on animals is due to the fact that different animals respond to the consumption of aflatoxin differently. It has also been observed that animals of the same species respond differently according to age, sex, and nutritional status. The acute toxicity of aflatoxin B_1 on various test animals is cited below. As can be seen, a wide range of LD_{50} value exists (Lillehoj and Zuber, 1975). The LD_{50} value represents a single oral dose.

Species	LD_{50} (mg/kg)
Rabbit	0.3-0.5
Rainbow trout	0.8
Dog	1.0
Monkey	2.2
Mouse	9.0
Rat	5.5-18.0

Repeated exposure of animals to aflatoxin over long periods of time frequently results in the formation of liver tumors. To date, aflatoxin is probably the most potent hepatocarcinogen known. Rainbow trout are extremely sensitive to liver tumor development from aflatoxin ingestion. In a study where trout were fed as little as 0.5 ppb aflatoxin B_1 in their diet for several months, 32% of the trout developed cancerous tumors (Wolan and Newberne, 1967).

Exposure to aflatoxin almost always occurs via ingestion of contaminated food. Contamination of food and/or grain crops occurs following infection by *A. flavus*. *Aspergillus flavus* infection of food and grain, especially corn, will ultimately lead to the production and excretion of aflatoxin. Infection of corn by *A. flavus* can occur in either pre- or post-harvest. Pot-harvest infection has been studied for many years. Conditions under which post-harvest infection and aflatoxin production occur are well defined. Pre-harvest infection and aflatoxin contamination were first documented in the mid-seventies (Lillehoj et al., 1976; Wilson et al., 1979; Zuber et al., 1976). Since that time, numerous other studies have demonstrated that pre-harvest infection is generally a more severe problem than post-harvest problems.

The primary source of inoculum for pre-harvest field infection of corn is assumed to be the soil. Although no data exists to document this theory, spores of *A. flavus* in the soil could be carried by wind or insects to the infection site of the field crop. Several previous studies have shown that the soil can harbor significant numbers of *A. flavus* propagules. Bell and Crawford (1967), using a Botran-amended media, found *A. flavus* soil populations to be as high as 2×10^4 propagules^{-1} g soil. Similarly, Angle and Wagner (1981) reported that soils cultivated to corn may contain populations of approximately 2×10^3 propagules^{-1} g soil. Other studies, however, have reported that soils contain few, if any, *A. flavus* propagules. A study by Clevstrom and Ljunggren (1984) failed to detect *A. flavus* in any of the soils assayed. Angle and Wagner (1981) also reported that some soils may contain very low numbers of *A. flavus*. Under optimum conditions, *A. flavus* conidia (Angle et al., 1988) and sclerotia (Wicklow, 1987) can survive in soil for at least a period of one year. These

structures can germinate in the spring, producing great numbers of additional conidia. This allows the infection cycle of corn to continue from one year to the next.

Of all crops contaminated with aflatoxin, contamination of corn is by far the most economically important. In the U.S., for example, during the years, 1977 and 1982 and most recently in 1988, more than 90% of all corn samples assayed from the Southeast U.S. were contaminated with aflatoxin. In Haiti, Castor et al. (1987) reported that sixty-nine percent of 268 samples of corn collected from the retail market in January, July, and October harvests were contaminated with aflatoxin.

Aflatoxin contamination is also a severe problem in the Philippines. Country-wide aflatoxin surveys have been conducted by local researchers (Santamaria et al., 1972; Salamat et al., 1982). The data they report indicate that most of the corn samples analyzed in the Philippines contain aflatoxin. See the following table for aflatoxin concentrations in various corn samples collected from the Philippines.

Incidence and range of aflatoxin in corn collected in the Philippines.

Sample	Incidence (%)	Aflatoxin (ppb)
Raw - whole	88 (360/407)[a]	3 - 1491
Raw - ground	95 (305/322)	3 - 510
Other products	59 (160/279)	3 - 735

[a] No. positive samples/total no. samples

In the Philippines, corn is reported to be one of the most heavily contaminated commodities and is known to contain high levels of aflatoxins under natural conditions (Garcia and Ila, 1986). On the Mindaneo and Cebu islands of the Philippines, corn and corn

products showed 100% aflatoxin contamination. Santamaria et al. (1972) further reported that corn grown in the Visayas and Mindanao regions contained more aflatoxin than those grown in Northern Luzon. This finding was confirmed by Tiongson and Gacilos (1986), who reported that corn with non-tolerable levels of aflatoxin was more prevalent in samples originating from Southern Mindanao (74%) than in those from Cagayan Valley (51%). This observation further confirms previous studies by Jayme et al. (1975), who reported that incidence of aflatoxin was higher in corn from the Southern Mindanao area than corn produced in the Cagayan Valley.

Other Philippine studies conducted at the Food and Nutrition Research Institute (FNRI), spearheaded by Dr. J.B. Jayme in 1976, include the possible relationship between the consumption of aflatoxin-contaminated corn by Philippinos and the development of primary liver cancer. It has been reported that the incidence of primary liver cancer by region is directly related to the per capita consumption of aflatoxin contaminated corn. This underscores the urgency of containing the aflatoxin problem.

5. Methods and Results:

Project 1. Determination of the heterokaryon compatibility group pattern in a field containing an endemic population of *Aspergillus flavus*.

A. METHODS

Land preparation, planting and layout. The experimental area was prepared following conventional methods of land preparation for upland areas. Fields were plowed three times and harrowed once. The corn variety used was IESCnl. This is an open pollinated yellow corn cultivar and the most widely planted cultivar in Isabela. It was planted on 1.5 m centers with furrows approximately 10 cm deep. Seeds were drilled on hills, and plants were thinned to four plants per 25 cm apart after 2 weeks.

A randomized complete block design with 4 treatments and 5 replications was used. Treatments were insecticide application, application of *A. flavus*, application of both insecticide and *A. flavus*, and an untreated control. Each replicate plot was split with one-half the plot receiving an additional treatment -- wounding of ears. Replicate plots were 6 m long and 16 rows wide. Only the middle 3 meters of both rows 3-6 and 11-14 were treated in each plot with one set of rows (randomly selected for each plot) also receiving the wounding treatment.

Application of *A. flavus* inoculum. Whole corn (500 g) in a 1-liter bottle was adjusted to 30% moisture, autoclaved, then inoculated with 1 ml 10^8 spores/ml *A. flavus* Min1A_2. Min1A_2 was isolated from field soil collected in Mindanao and consistently produced over 100 µg/kg aflatoxins in liquid fermentation (Cotty and Bayman, 1993). After inoculation, the corn was incubated stationary (30 ± 1°C) for 7 days. Every 48 hours the

bottles were shaken vigorously by hand to reduce sporulation and prevent kernel clumping. Colonized corn (250g/plot) was applied to the soil surface 20 days after emergence (DAE).

Insecticide application. Insecticides were only applied to replicate plots of the insecticide treatment. Nuvacron (monocrotophos) was applied 20 DAE. Other insecticides (Decis; Furadan [Carbofuran]; Cymbush [Cypermethrin]) were applied when insect damage to the developing crop was observed. Insecticides were applied 4 times to the dry season test and 8 times to the wet season test.

Wounding of developing ears. At 50% silking, ears were wounded on two opposing sides. Wounds were formed by piercing the developing kernels through the husks with improvised nailboards (20 cm x 8 cm). The board contained eight No. 2 nails spaced about 2 cm apart. Five ears on each of four rows were wounded in each replicate plot (20 plots and 400 ears total per test).

Harvesting, drying, and shelling. At maturity, 20 wounded ears and 20 ears not wounded were harvested from appropriate sides of each replicate split plot. Harvested ears were initially sun-dried on a cement pavement for 24 hours. Subsequently, husks were removed and extent of physical ear damage was estimated visually as the percent of damaged kernels per ear with three categories (<5%; about 10%; about 20%). During shelling, kernels were divided into two subgroups: one with damaged and immediately adjacent kernels were combined, and sound kernels separated from damaged kernels by at least 2 kernels were combined. These two groups of kernels were analyzed separately. In wounded treatments, kernels damaged by piercing with a nailboard were isolated for the sounded category. In

treatments without wounding, kernel damage by natural agents (rodents, birds, mites and insects) were used. Most ears had some damaged kernels.

Assessment of kernel infection. In each test, 400 kernels of each subgroup from each replicate split plot (32,000 total kernels) were assessed for kernel infection. Kernels were shaken by hand for 5 min in 1% NaOCl, washed 3 times with sterile, distilled water; blotted dry and plated on Malt Salt Agar (MSA) at 25 seeds per plate. The plates were incubated at room temperature (30 ± 1°C) for 7-10 days and visually assessed for *A. flavus* growth.

Aflatoxin analyses of corn. Aflatoxin B_1 content of the corn was determined using the previous modifications to methods of the Association of Official Analytical Chemists (Cotty, 1989; Stoloff and Scott, 1984).

All kernels not used to assess kernel infection (200 to 700 g) were pooled and ground using a manual grinder. After mixing, a 50 g subsample was taken and added to 200 ml 85% aqueous acetate. The mixture was shaken for 15 sec, allowed to settle overnight, and filtered through No. 4 Whatman paper. The filtrate (100 ml) was mixed with 20 ml of a solution of zinc acetate and aluminum chloride [1.1 M $(CH_3COO)_2Zn$ and 0.04 M $ALCl_3$], together with 80 ml of water and 5 g of celite (Fluka 545). The mixture was shaken, left to settle for 30 min to 1 hr, and passed through No. 4 Whatman filter paper. The filtrate (100 ml) was extracted twice with 25 ml of methylene chloride. Extracts were combined, passed through 3 g anhydrous sodium sulfate (Na_2SO_4) and evaporated to dryness. Residues were transferred to a vial (8 ml) using approximately 4 ml methylene chloride, was evaporated to dryness, and stored dry in the freezer until assayed by TLC.

Thin-layer chromatography (TLC). TLC plates (20 x 20 cm) coated with 0.5 mm Silica gel GF (Analtech Uniplates No. 7020111) were reactivated for 1 hr at 105°C and stored in a desiccating cabinet. Initially, residues were solubilized in 1.0 ml methylene chloride and shaken uniformly with a vortex mixer. Plates were spotted with both five and ten microliters (µl) each of the sample extract, internal standard (sample spotted over the standard), and standard. Spotted plates were developed in diethyl ether: methanol: water (97:2:1, v/v) until the solvent front had transversed about 8-10 cm from the origin. Plates were removed from the developing tank and air-dried for about 2 min. Sample fluorescence was compared to the standards under long-wave ultraviolet light viewer. The amount of aflatoxin B_1 was estimated based on relative fluorescence. If sample fluorescence exceeded that of the standard, the sample was diluted appropriately. Each plate containing samples with aflatoxin B_1 was sprayed with 25% sulfuric acid (H_2SO_4) and fluorescence was reexamined in order to confirm presence of aflatoxin B_1 (Scott, 1990).

B. RESULTS

In both dry season and wet season tests, in all treatments, at least 1% of kernels were infected with *A. flavus* (Table 1). This held for untreated controls and both damaged and sound kernels. These results are consistent with those of Ilag, where during a two-year Philippine-wide survey of infection of maize in the field, kernel infection rates ranged from 1.66 to 52% (Ilag). Ilag's survey did not include aflatoxin analyses, but in subsequent work, 60 to 95% of Maize products from the Philippines were found to contain aflatoxins. Although aflatoxin B_1 levels detected in the current study significantly differed among

treatments, in all cases aflatoxin B_1 levels that would be unsafe for human consumption occurred (Jayme, Cotty, et al.). The consistency and severity of the observed contamination, particularly in treatments without inoculation, differ markedly from typical field tests in temperate regions with lower endemic contamination levels (Widstrom, Cotty, et al.; Jones, Lillehoj, McMillan).

Visual assessment of kernel damage was imprecise (estimated to <5% , about 10%, or about 20%), but it revealed greater damage in the wet season than in the dry season. In the dry season, although damage was present in all replicates of all treatments, in no case was damage greater than 5% of kernels. In the wet season, treatments without wounding also had less than 5% damaged kernels, but all replicates of treatments receiving wounding had 10 to 20% kernel damage. Kernel damage in treatments without wounding may be attributed to insects, rodents, and birds. These agents typically cause some damage in developing corn, and in the Philippines their incidence is more severe in the rainy season (Lillehoj et al., 1976; and Benigno, 1980, 1983).

The extent of ear damage is visually evident and higher in all the wounded treatments than the treatments not wounded for both the dry and wet cropping seasons of 1992-1993. Higher values (about 20%) were observed during the wet season than the dry season (<5%) for which data is not shown. Kernel damage in the treatments not wounded could be caused by the natural and common occurrence of insects, mites, rodents, birds and other pests of corn and apparently have been described previously by Lillehoj et al., 1976 and Benigno, 1983. Incidence of these pests was more severe and the wounding damage caused by them was remarkable during the rainy season (Benigno, 1980, 1983).

During the dry season, the degree of ear damage was generally directly related to the extent of *Aspergillus* kernel infection. Table 1 shows that kernel infection and aflatoxin content of seeds were consistently higher with wounding and often these differences were statistically significant (P = 0.05). Kernel infection increased when wounding and fungus applications were combined as compared to the wounded treatment alone. However, kernel infection did not significantly increase with fungus application in treatments without wounds. For damaged kernels directly adjacent to the wounds, highest infection (46%) was observed in the treatment with wounding, fungus application, but not treatment with insecticide. However, damaged kernels directly adjacent to the wounds were significantly reduced (30%) when insecticide was applied. Lower kernel infection with insecticide application could be attributed to the reduction in insect activity, which consequently minimizes the dispersion of the fungal mycelia/spores through the insect body (Diener et al., 1987). McMillan et al. (1978) also indicated a direct correlation between insect damage and *Aspergillus* kernel infection, although later a study by the same authors (McMillan et al., 1985) revealed that insects were not solely responsible for the infection observed. Wounded treatment without the fungus and insecticide applied had a kernel infection of 29% which was significantly reduced to 12% when insecticide was applied. Results suggested that, even without inoculation, *A. flavus* kernel infection occurred. This finding could be attributed to the high indigenous population levels of *A. flavus* in the soil. Similarly, this could be explained further by the ubiquitous nature of *Aspergillus flavus* group species.

Generally, the extent of *Aspergillus* kernel infection of damaged kernels directly adjacent to the wound among the not wounded treatments did not vary significantly with each other. Much lower values compared to the wounded treatments were observed (Table 1). Kernel infection among these treatments could be attributed to the usual ear damage created by naturally and commonly occurring pests of corn, like corn borer, earworm, mites, etc. (Fennell et al., 1975). These vectors provided access for the subsequent establishment and invasion of the fungus. Windels et al. (1975) reported that the ear region of the corn leaves were preferred for oviposition of corn borer egg masses. The larvae from these eggs feed on silk and quickly enter the ear, making chemical control ineffective and more difficult. This supports the present observation in that the treatments not wounded, both in damaged kernels directly adjacent to the wounds and sound kernels separated from damaged kernels by at least two kernels, did not vary significantly.

Another important role of insects in this study is that they serve as a vector in the infection process. Insects carry and spread the fungal spores from the point of entry and from kernel to kernel within the ear as exhibited by those not wounded, without insecticide and fungus applications (Table 1). Likewise, sound kernels separated from damaged kernels by at least two kernels also were infected, although the extent of *A. flavus* kernel infection is less compared to those damaged kernels directly adjacent to the wounds.

In the wet season crop, extensive kernel rot occurred, particularly in wounded treatments. However, most infection was caused by the most frequently isolated rotting fungi, *Fusarium moniliforme*. Thus, lower *Aspergillus* kernel infection occurred in the wet season than in the dry season, and often both wounding and fungus applications did not

significantly increase either kernel infection by *A. flavus* or aflatoxin content (Table 1). The findings of Zummo et al. (1992) supported the present observation that lower infection in *Aspergillus*-inoculated ears was exhibited in the presence of *Fusarium moniliforme*, indicating interference of the latter fungus in the infection process of the former. Furthermore, the difference may be attributed to the effects of varying environmental conditions prevailing during the growing season. Jones et al. (1981) observed higher infection in corn that silk during periods of high airborne spore load. Lower *A. flavus* kernel infection observed may also be attributed to lower inoculum density in the corn silk as raindrops can detach and wash away the fungal spores from the host plant in addition to wind dispersion (Fitt et al., 1989). In addition, competition by other fungal species may also play a role as lower temperature during the rainy season is not conducive to *Aspergillus* growth (Jones et al., 1980).

Table 2 shows the average percent response to treatments. During the dry season, there was an increase of 676% and 594% of *A. flavus* kernel infection in wounded and sound kernels, respectively. Insecticide application drastically decreased both kernel infection and aflatoxin content in the treatments not wounded. The insecticide might have protected the wounds and eliminated the insect vectors/carriers that disperse the fungal spores. However, insecticide application in the treatments not wounded generally did not reduce kernel infection and aflatoxin content in wounded and sound kernels, both in dry and wet seasons. It is possible that the insecticide applied did not kill all the insects in the field; for it might be specific only to certain insects. Furthermore, other pests, like rodents, birds and mites, which are naturally occurring and common in typical corn growing conditions in the Philippines,

might have contributed in the wounding injury and predisposed the plant tissue for higher kernel infection and aflatoxin contamination. Fungus application seems not to tremendously affect percent kernel infection and aflatoxin content both in wounded and sound kernels during the dry and wet seasons. This could be attributed to sufficient indigenous *A. flavus* inoculum levels present in the soil used for experimental areas.

Wounding of developing ears by rodents, insects, mites and birds is common in the Philippines and the current study shows such wounding plays an important role in predisposing corn to *Aspergillus* infection and aflatoxin contamination under the typical local growing conditions. Insect activity and inoculum density of the fungus greatly influence the extent of contamination in the crop at harvest, but the effects of these parameters are complicated. Experimental results revealed that wounding and insects play an important role in preharvest kernel infection by *A. flavus* and the subsequent aflatoxin contamination. Thus, the present study suggests aflatoxin contamination of corn in the Philippines may be reduced by efforts directed towards minimizing wounding of the developing crop.

Project 2. Establishing a direct relationship between soil populations of *Aspergillus flavus* and aflatoxin contamination of corn.

A. METHODS

Land preparation, planting and lay-out. The experimental area was prepared following conventional methods of land preparation for upland areas. Fields were plowed three times and harrowed once. The corn variety used was IESCnl, an open pollinated yellow corn cultivar and the most widely used cultivar in Isabela. It was planted on 75 cm centers with furrows approximately 10 cm deep. Seeds were drilled on hills and plants were thinned to four plants per 25 cm apart after 2 weeks.

A randomized complete block design (RCBD) with 6 treatments and 5 replications was used. Treatments consisted of two types of inoculation (basal and spray), two plant stages when fungus was applied (20 DAE and 50% silking stage), and two types of wounding simulation (insect and rat damage). Replicate plots were 4.5 m x 6 m. Only the inner 4 rows (3.5 m long) of each plot or a total inoculated area of 3.5 m x 3.0 m (10.5 m^2) were used.

Inoculum preparation and inoculation. Whole corn (250g) in a 6 x 8 inch heat-resistant plastic bag was adjusted to a 30% moisture, autoclaved, then inoculated with 1 ml 10^6 spores/ml *A. flavus* Min1A_2. Isolate Min1A_2 was isolated from corn field soil collected in Mindanao and consistently produced over 100 µg/kg aflatoxins in liquid fermentation (Cotty and Bayman, 1993). After inoculation, the corn was incubated stationary (30 ± 1°C) for 7 days. Every 48 hours the plastic bags were shaken uniformly by hand to reduce sporulation and prevent clumpings of kernels.

For basal inoculation, colonized corn (250g/plot) was spread on the soil surface 20 days after emergence (DAE). For spray inoculation, 10^6 spores/ml *A. flavus* MinlA$_2$ suspension was prepared by growing the fungal isolate in 5/2 agar (5% V-8 vegetable juice, 2% agar, pH 5.2) for 5-7 days. Spores were harvested, adjusted to a 10^6 spores/ml concentration and 0.01% Tween 80 was added to the suspension as a surfactant. Inoculation was done by spraying the wounded corn ear until run-off using an atomizer.

Insecticide application. Insecticides were applied to replicate plots when deemed necessary. Nuvacron (monocrotophos) was applied 20 DAE. Other insecticides (Decis; Furadan [Carbofuran]; Cymbush [Cypermethrin]) were applied when insect damage to the developing crop was observed. Insecticides were applied 4 times to the dry season test and 8 times to the wet season test.

Wounding simulation of developing ears. Simulation of two types of wounding (insect and rat damage) was done at approximately 50% silking stage of corn. For simulated insect damage, ears were wounded on two opposing sides. Wounds were formed by piercing the developing kernels through the husks with improvised nailboards (20 cm x 8 cm). The board contained eight No. 2 nails spaced 2 cm apart.

Simulated rat damage was done by cutting a portion of the husk (8 x 5 cm) in one side, exposing approximately one-third of the developing corn cob and scratching the developing ears twice with improvised nailboards. The inoculum was sprayed primarily to the wounded portion of the ear. For replicated plots that were basally applied with corn colonized with 10^6 spores/ml *A. flavus* suspension, at 20 DAE, no more fungus was applied

at 50% silking stage of corn ears, but wounding alone was done. Five ears on each of four rows were wounded on each replicate plot (20 plots and 400 ears total per test).

Harvesting, drying, and shelling. At maturity, all ears within the inoculated area were harvested. Five ears of approximately similar sizes were selected from each row and placed in individual net bags with a total of 20 ears sampled per plot. Harvested ears (28% moisture) were initially sun-dried on a cement payment for 24 hours. Subsequently, husks were removed and the extent of physical ear damage was estimated visually as percent damaged kernels per ear with three categories (<10%; about 50%, >90%). Ears were further dried in a gas operated dryer at 60° C to a moisture content of 14-15% moisture.

Dried kernels were shelled manually, and during shelling, kernels were divided into two subgroups (one with damaged and directly adjacent to damaged kernels which were combined and sound kernels which were separated from damaged kernels by at least 2 kernels) which were combined. These two groups of kernels were analyzed separately. In wounded treatments, kernels damaged by piercing with a nailboard were selected for simulated insect damage, while kernels damaged by scratching with a nailboard were used for simulated rat damage. In treatments without wounding, kernel damage by natural agents (rodents, birds, mites, and insects) were used. Most often ears had some damaged kernels.

Assessment of kernel infection. In each test, 400 kernels of each subgroup from each replicate plot (24,000 total kernels) were assayed for kernel infection. Approximately 100 corn seeds were surface sterilized with 1% NaOCl (using Zonrox brand with 5.25% active ingredient) for 5 min, rinsed three times with sterile distilled water, blotted dry and

plated on Malt Salt Agar (MSA) at 25 seeds per plate. The plates were incubated at room temperature (30 ± 1°C) for 7-10 days and visually assessed for *A. flavus* growth.

Aflatoxin analysis of corn. Aflatoxin B_1 content of corn was determined using previous modifications to methods of the Association of Official Analytical Chemists (Cotty, 1989; Stoloff and Scott, 1984).

All kernels not used to assess infection (200 to 700 g) were pooled and ground using a manual grinder. After mixing, a 50 g subsample was taken and added to 200 ml 85% aqueous acetate. The mixture was shaken for 15 sec, allowed to settle overnight, and filtered through No. 4 Whatman paper. The filtrate (100 ml) was mixed with 20 ml of a solution of zinc acetate and aluminum chloride [1.1 M $(CH_3COO)_2Zn$ and 0.04 M $ALCl_3$], together with 80 ml of water and 5 g of celite (Fluka 545). The mixture was shaken, left to settle for 30 min^{-1} hr, and passed through No. 4 Whatman filter paper. The filtrate (100 ml) was extracted twice with 25 ml of methylene chloride. Extracts were combined, passed through 3 g anhydrous sodium sulfate (Na_2SO_4) and evaporated to dryness. Residues were transferred to a vial (8 ml) using approximately 4 ml methylene chloride, evaporated to dryness, and stored dry in the freezer until assayed by TLC.

Thin-layer chromatography (TLC). TLC plates (20 x 20 cm) coated with 0.5 mm Silica gel GE (Analtech Uniplates No. 7020111) were reactivated for 1 hr at 105°C and stored in a desiccating cabinet. Initially, residues were solubilized in 1.0 ml methylene chloride and shaken uniformly with a vortex mixer. Plates were spotted with both five and ten microliters (μl) each of the sample extract, internal standard (sample spotted over the standard), and standard. Spotted plates were developed in diethyl ether: methanol: water

(97:2:1, v/v) until the solvent front had transversed about 8-10 cm from the origin. Plates were removed from the developing tank and air-dried for about 2 min. Sample fluorescence was compared to the standards under long-wave ultraviolet light viewer. The amount of aflatoxin B_1 was estimated based on relative fluorescence. If sample fluorescence exceeded that of the standard, the sample was diluted appropriately. For each sample containing aflatoxin B_1, aflatoxin identification was confirmed by spraying the TLC plate with 25% sulfuric acid (Scott, 1990).

B. RESULTS

The extent of ear damage was relatively rated and is highest in treatments with simulated rat damage regardless of the type of inoculum used, on what stage of plant growth it was inoculated, and what season the test was conducted. Higher values (>90%) were observed during the wet season than the dry season (<10%) in which data is not shown (Table 3). Similar results were obtained during the two cropping seasons but with a higher degree of damage observed during the wet season trial. This indicates that injury and deterioration of kernels from ears by simulated wounding were further enhanced by the prevailing environmental conditions in the wet season. Kernel damage in the treatments not wounded could be caused by the commonly occurring pests of corn which were not totally eliminated by the insecticide application. Incidence of these pests was more prevalent and severe, and the wounding damage caused by them was remarkable during the rainy season (Benigno, 1980; 1983).

In the dry season crop, the highest infection (89%) and aflatoxin content (160 ppm) was exhibited in the simulated rat damage sprayed with 10^6 spores/ml *A. flavus* Min1A_2 at silking stage. This was followed by the same type of wounding inoculated on the soil surface with colonized corn with 10^6 spores/ml *A. flavus* at 20 days after emergence (20 DAE) with 57% kernel infection and 152 ppm aflatoxin content on wounded kernels. Generally, kernel infection and aflatoxin content of seeds were significantly higher with any type of wounding compared to the treatments not wounded, and often these differences were statistically significant (P=0.05). The simulated corn borer damage values were higher compared to the treatment control not wounded; however, they were significantly different only in the aflatoxin content for damaged kernels directly adjacent to the wounds and sound kernels separated from damaged kernels by at least two kernels when sprayed at the silking stage. *Aspergillus* infection and aflatoxin content in the simulated corn borer damage, with colonized corn applied in the soil at 20 days after emergency and 10^6 spores/ml *A. flavus* sprayed at 50% silking stage of corn, did not vary significantly. Fennell et al. (1975) cited varying levels of fungal infection in corn damaged by different types of pests like earworm, borer, bugs, mites and beetles with kernels from insect-damaged ears showing a distinctly higher occurrence of fungi than those from insect-free ears.

Sound kernels separated from damaged kernels by at least two kernels were also observed to have some degree of infection and aflatoxin content (Table 4), although the extent is generally lower compared to that of damaged kernels directly adjacent to damaged kernels. This indicates that *A. flavus* kernel infection is first localized near the infection site and later spreads to the surrounding kernels, as well as by either wind, insects or other pests.

Similar phenomenon was observed by Fennell et al. (1975), where fungal infection remained localized in the insect feeding area, but later spread, as insects and other pests' vectors thrive and disseminate fungal mycelia and spores. Wicklow et al. (1988) further observed such a phenomenon, wherein uninjured and sound kernels away from the wounded areas became infected by *A. flavus*. Cottonseed from non-damaged locks may be an important component of the overall aflatoxin contamination problem (Cotty and Lee, 1989).

During the wet season crop, extensive kernel rotting occurred and was relatively higher compared to that of the dry season crop. However, kernel rot was caused by the most frequently isolated rotting fungi-*Fusarium moniliforme*. This indicates that, during the wet season, *Aspergillus* growth and infection were not favored by such climatic and environmental conditions. Wounding damage created on the developing ear may also predispose corn to invasion by other fungi or antagonists, so that competition would be an important factor for the successful establishment of *Aspergillus flavus* species. Other fungal genera observed to be associated with insect damage were *Penicillium*, *Rhizopus*, and *Syncephalastrum* (Fennell et al., 1975). The present study observed that *Fusarium moniliforme* is the most prevalent and frequently isolated rotting fungi in *Aspergillus* infected corn kernels. Zummo and Scott (1990) also reported frequent isolation of *Fusarium moniliforme* in kernels from ears inoculated with *Aspergillus flavus*. The same authors (Zummo and Scott, 1992) observed an apparent inhibition of *Aspergillus* kernel infection leading to reduced aflatoxin contamination by *Fusarium moniliforme*. The variations in *A. flavus* kernel infection and aflatoxin content observed between the dry and wet cropping seasons could also be attributed

to other factors, like planting and harvest dates (Jones et al , 1981) and cultural practices (Angle et al , 1982)

Results from this study suggest that the different wound types created by common agricultural pests like rats and insects have varying influence on kernel infection and subsequent aflatoxin formation in preharvest corn Apparently, the more extensive the wounding of kernels, the greater vulnerability to aflatoxin contamination The influence of each wound type is also affected by the prevailing climatic, weather, and environmental conditions within the field during the crop growing season

Project III. Determining the importance of insects and wounding to kernel infection of *A. flavus* and what types of wounds predispose corn to infection. Draft manuscript in preparation.

6. Impact, Relevance and Technology Transfer:

The findings of this study show that the soil is a significant source of inoculum for corn is impoarant in that it opens up a whole new area for controlling the initial infection of corn. Currently, we attempt to remediate contaminated corn following infection and aflatoxin contamination. These procedures, however, are too expensive for lesser developed countries. From the current study, it is suggested that if the original source of inoculum can be controlled in the soil or if the transmission from soil to corn is reduced it should be possible to reduce the aflatoxin concentration in corn. Methods for controlling the inoculum are relatively inexpensive and are amendable for use in lesser developed countries. Thus, the next stage of the research should be to investigate methods for reducing the inoculum load in soil or for controlling insects that are responsible for transmission.

An important result of this project is that modern equipment is now available in the Philippines for the measurement of aflatoxin in food. Prior to this project, all analyses for aflatoxin were preformed on relatively crude instruments that were less than accurate. Dr. Rosalinda Garcia now has the most modern aflatoxin analysis lab in the country and has already become an important source of food evaluation for the entire region.

7. Project Activities/Output:

Three journal publications should be ready for submission in early 1996. The results of the study were presented at the World Mycology Conference in Vancouver, British Columbia in August 1994. All three principal investigators attended this meeting. Dr. Garcia spent approximately six months at the USDA-ARS lab in New Orleans learning modern techniques for aflatoxin analyses. She also became familiar with methods for typing strains of *Aspergillus*. Presently, she is the only scientists in the Pacific region with this capability.

8. Project Productivity:

All project goals were completed during the four year period.

9. Future Work:

At this time, no additional work is contemplated. Although the next step of the research could potentially lead to a reduction of aflatoxin in corn, few funds are presently available for continuation.

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Table 1. Influence of wounding, insecticide and fungus application on corn kernel infection by *Aspergillus flavus* and aflatoxin contamination.

Treatment[u]	Dry season[s]				Wet season[t]			
	Kernel Infection %[v]		Aflatoxin B_1[w] (ppm)		Kernel Infection %		Aflatoxin B_1 (ppm)	
	Wounded[x]	Sound[y]	Wounded	Sound	Wounded	Sound	Wounded	Sound
W + I + F	30 b[z]	11 b	58 bc	39 b	2 b	3 bc	25 abc	14 b
W + I - F	12 c	7 c	43 cd	30 c	9 a	3 b	27 ab	13 bc
W - I + F	46 a	30 a	88 a	77 a	11 a	3 bc	32 a	24 a
W - I - F	29 b	14 b	77 ab	64 a	4 b	2 bc	12 bdc	10 bc
NW + I + F	4 de	2 d	28 d	32 b	10 a	7 a	14 bcd	10 bc
NW + I - F	5 cde	3 cd	44 cd	39 b	8 a	6 a	10 bcd	8 bc
NW + I + F	10 cd	5 cd	22 d	23 bc	2 b	2 c	7 cd	11 bc
NW - I - F	2 e	2 d	18 d	10 c	2 b	1 d	4 d	5 c

[s] Dry season extended from Dec. 1992 to March 1993.
[t] Wet season extended from June 1993 to Sept. 1993.
[u] Treatments: W = wounded; NW = not wounded; I = insecticides applied; F = corn colonized by *A. flavus* applied.
[v] Each value is an average of 5 replications with 20 ears per replicate. Average of 400 kernels per replicate.
[w] Each value is an average of 5 replications with 20 ears per replicate. Kernels were pooled and ground using a manual grinder. After mixing, fifty grams subsamples were analyzed for aflatoxin.
[x] Wounded = damaged kernels and kernels directly adjacent to damaged kernels.
[y] Sound = sound kernels separated from damaged kernels by 1 or 2 kernels.
[z] Means in a column followed by a common letter are not significantly different at the 5% probability level by Duncan's Multiple Range Test (DMRT).

Table 2. Response to the main treatments, wounding; insecticide; and fungus application, averaged across subtreatments.

	Average response to treatment (%) [a]							
	Dry Season [b]				Wet Season [c]			
	Kernel infection (%)		Aflatoxin B_1 (PPM)		Kernel infection (%)		Aflatoxin B_1 (PPM)	
Treatment	Wounded [d]	Sound [e]	Wounded	Sound	Wounded	Sound	Wounded	Sound
Wounding	676[f]	594	282	285	249	158	314	176
Insecticide application	96	74	123	153	313	410	192	112
Wounded	53	42	61	48	119	129	152	95
Unwounded	138	107	184	257	508	690	233	130
Fungus application	236	172	107	136	124	140	167	180

[a] Average percent response across subtreatments (i.e., for wounding, the value is the average percent of the unwounded value observed in the corresponding wounded value averaged across all treatments: Wounded alone and not wounded, wounded plus insecticide and not wounded plus insecticide, wounded with fungus and not wounded with fungus, and wounded with insecticide and fungus and not wounded with insecticide and fungus).

[b] Dry season extended from December 1992 to March 1993.

[c] Wet season extended from June 1993 to September 1993.

[d] Wounded = damaged kernels plus kernels immediately adjacent to damaged kernels.

[e] Sound = sound kernels separated from damaged kernels by 1 or 2 sound kernels.

[f] Each value is the average response in four subtreatment comparisons. Each subtreatment was replicated 5 times.

Table 3. Establishing a direct relationship between soil populations of *Aspergillus flavus* and aflatoxin contamination of corn.

Treatment	Dry Season						Wet Season					
	Propagule Count After Treatment[1]	Relative Rating of Damaged Kernel[2]	Kernel Infection, %		Aflatoxin Contamination, ppm		Propagule Count After Treatment	Relative Rating of Damaged Kernel	Kernel Infection, %		Aflatoxin Content, ppm	
			KAW	SKW	KAW	SKW			KAW	SKW	KAW	SKW
1-10^6 spores/ml (spray)	17.4 c[5]	3.0 c	7.5 b	7.7 g	50 d	48 c	15.2 g	25.0 a	6.3 d	5.7 a	7.1 bc	7.5 ab
2-50g colonized corn (basal)	35.2 bc	3.3 b	16.5 ab	16.2 c	30 c	20 f	107.8 d	22.1 ab	5.0 c	3.0 c	10.4 ab	10.1 a
3-10^8 spores/ml (spray)	22.2 c	3.7 a	18.0 ab	9.3 f	54 c	28 c	28.0 f	12.4 c	14.0 a	5.1 b	8.5 abc	10.1 a
4-200g colonized corn (basal)	50.7 b	2.1 d	16.6 ab	12.2 c	82 b	68 b	265.2 b	19.8 abc	8.8 c	3.6 c	12.3 a	6.9 ab
5-10^{10} spores/ml (spray)	27.4 bc	1.5 f	15.2 ab	15.4 d	28 f	36 d	141.3 c	15.5 bc	4.0 f	5.0 b	9.6 ab	10.7 a
6-400g colonized corn (basal)	99.4 a	2.1 d	24.7 a	22.3 a	176 a	148 a	316.7 a	19.6 abc	10.8 b	5.4 a	5.6 bc	4.7 ab
7-Control (uninoculated; wounded)	18.8 c	1.8 e	10.7 ab	17.0 b	28 f	16 g	40.5 e	18.1 abc	4.0 f	3.3 d	3.6 c	1.6 b

[1] Each value is an average propagule count of 4 plate counts per replication.
[2] Each value is an average of 5 replications with 20 ears per replicate.
[3] Each value is an average of 5 replications with 20 ears per replicate. Average of 400 kernels per replicate.
[4] Each value is an average of 5 replications with 20 ears per replicate. Samples were pooled and 50 grams were analyzed for aflatoxin.
[5] Means in a column followed by a common letter are not significantly different at 5% probability by DMRT.

Table 4. Determining the type of wounds that predispose corn cobs to *Aspergillus flavus* kernel infection and aflatoxin contamination.

Treatment	Dry Season[t]				Wet Season[u]			
	Kernel Infection %[v]		Aflatoxin B_1[w] (ppm)		Kernel Infection %		Aflatoxin B_1 (ppm)	
	Wounded[x]	Sound[y]	Wounded	Sound	Wounded	Sound	Wounded	Sound
Simulated corn borer damage (20 DAE; basal)	38[z] bc	24 b	32 bc	24 cd	11 bc	3 c	14 b	6 c
Simulated rat damage (20 DAE; basal)	57 b	22 b	152 a	72 b	23 b	16 ab	31 a	27 a
Not wounded (20 DAE; basal)	18 d	5 c	16 c	4 d	20 bc	4 c	34 a	25 ab
Simulated corn borer damage (silking; spray)	31 c	16 bc	74 b	60 bc	18 bc	14 b	9 b	9 c
Simulated rat damage (silking; spray)	89 a	54 a	160 a	114 a	35 a	24 a	12 b	6 c
Not wounded (silking; spray)	22 d	9 bc	26 c	14 d	9 c	5 c	11 b	12 bc

[t] Dry season extended from Dec. 1992 to March 1993.
[u] Wet season extended from June 1993 to Sept. 1993.
[v] Each value is an average of 5 replications with 20 ears per replicate. Average of 400 kernels per replicate.
[w] Each value is an average of 5 replications with 20 ears per replicate. Kernels were pooled and ground using a manual grinder. After mixing, fifty gram subsamples were analyze for aflatoxin.
[x] Wounded = damaged kernels and kernels directly adjacent to damaged kernels.
[y] Sound = sound kernels separated from damaged kernels by at least 2 kernels.
[z] Means in a column followed by a common letter are not significantly different at the 5% probability level by Duncan's Multiple Range Test (DMRT).